REMARKS UNDER 37 CFR § 1.111

Formal Matters

This paper is responsive to the Office Action dated October 3, 2002 (Paper No. 5), which is the first action on the merits of the application.

Claims 31-60 were previously pending in the application, and under examination.

As a result of entering this Amendment, certain claims are amended, and claims 61-78 are added. The new claims cover methods for stimulating an anti-tumor immune response or treating a neoplastic disease, and therefore fall within the same category as the claims previously pending. Accordingly, claims 31-78 are now under examination.

No new matter has been added to the disclosure as a result of entering these amendments.

Reconsideration and allowance of the application is respectfully requested.

Interview:

The applicants wish to thank Examiner Christopher Yaen for a constructive and helpful telephone interview with Carol Francis and Michael Schiff on February 27, 2003. Recommendations made by Dr. Yaen are incorporated into this response.

Amendments to the claims:

No new matter is added to the disclosure as a result of entering this paper into the application file. Support for the amendments and the new claims can be found throughout the application as originally filed, including the following:

Claim 31:	Claims 31 and 45 as previously presented; page 31 lines 17-19
Claim 45:	Page 59, lines 14-23
Claim 50:	Claims 50 and 45 as previously presented; page 31 lines 17-19
Claim 55:	Page 41, lines 8-10
Claim 56:	Page 41, lines 20-21
Claims 61-63:	Claim 32 as previously presented
Claim 64:	Claims 31 and 36 as previously presented
Claim 65:	Claim 32
Claim 66:	Claim 34
Claims 67-71:	Claims 38-41

Claims 72-73:	Claims 45 and 46; claim 31
Claims 74-76:	Claims 51-53
Claims 77-78:	Claim 31

Reference to the cytokine-producing cell has been changed from the wording originally presented (a genetically altered cell) to a cell that expresses a cytokine from a recombinant polynucleotide. This has been done to facilitate the wording of the rest of the amended claims. The skilled reader will appreciate that the polynucleotide referred to can be a vector that is resident in the cell (such as an adenovirus: page 30, lines 4-18), or a recombinant nucleic acid introduced into the genome (such as by a retroviral vector: page 30, line 19 to page 31, line 16). The cell can be the same cell initially transfected with the vector, thereby containing the recombinant polynucleotide, or a progeny thereof that has inherited the recombinant polynucleotide.

Rejections under 35 USC § 112 ¶ 2:

Various claims were rejected under §112, ¶2. Each of these rejection is addressed below.

Claim 34 stands rejected as indefinite for alternately referring to "tumor" and "cancer". Of course, not all tumors are cancerous. This claim has now been amended to use the term "tumor" throughout.

Claim 38 stands rejected as indefinite for referring to a composition "effective in treating" the disease or eliciting an immunological response. Applicants respectfully disagree. This phrase is common terminology in issued U.S. patents, and will be clear to the clinician or immunologist managing the subject's care. Treatment is defined in the specification on page 21, and desirable outcomes of treatment are exemplified on pages 34-38. Accordingly, the term complies with the requirements of § 112 ¶ 2. The actual dose and formulation of the composition will depend on the nature of the tumor and condition of the patient, and will be optimized empirically. This is an objective of Phase II and Phase III human clinical trials.

Claim 50 stands rejected as unclear for referring to a level "sufficient" to stimulate an immune response. This claim has been amended to refer to the composition as a whole as "effective" in stimulating an immune response.

Claim 38 and 50 stand rejected as unclear for referring to a "tumor associated antigen". Applicants respectfully disagree. The term is extensively defined and described on page 21,

lines 5-15. As exemplified elsewhere in the specification, the antigen may be present in the composition expressed on a cell, or obtained from a cell that expressed it.

Claims 55 and 56 stand rejected as unclear for referring to a primary and secondary immune response. The wording has now been changed to refer to priming, and boosting or maintaining an immune response. The skilled reader will appreciate that these terms are substantially the same as the previous terms.

The amendments made under this section do not narrow the claim scope. Accordingly, these claims protect equivalents by an extent to which they are otherwise entitled.

Withdrawal of these rejections is respectfully requested.

Rejection under 35 USC § 112 ¶ 1:

All claims under examination stand rejected under this section as being enabling for administering membrane M-CSF expressing cells, but not cells expressing cytokines that are usually secreted.

Applicants respectfully disagree. The Office Action seeks to limit coverage to the working examples. Of course, this is not the legal standard. The specification need not provide actual data for all possible species that fall within the genus. It is sufficient for the specification to provide procedures for carrying out the invention, which in combination with reagents and procedures otherwise known in the art, allow someone skilled in the art to practice the claimed invention without undue experimentation.

Page 26, line 24 to page 27, line 20 provides an extensive discussion of membrane-associated cytokines, and how cytokines that are normally secreted can be altered so as to be expressed in a membrane-associated form. For example, it tells the reader that cells can be genetically altered with a vector containing a cytokine encoding region and a transmembrane region in the same open reading frame. The transmembrane region may be modeled on other known transmembrane proteins, or an artificially designed polypeptide segment with a high degree of lipophilicity.

Accompanying this response is an article by W. Soo Hoo et al., entitled *Tumor cell surface expression of GM-CSF elicits antitumor immunity and protects from tumor challenge in the P815 mouse mastocytoma tumor model.* J. Immunol. 162:7343-7349, 1999. The authors followed the procedure provided on page 27 of the specification using the pHOOK-1 plasmid

vector, commercially available from Invitrogen. The vector as sold apparently already contains the PDGF receptor transmembrane domains, and restriction nuclease sites for inserting a heterologous extracellular domain. The authors of this paper ligated the GM-CSF encoding region into the vector using standard techniques. There is no indication that the procedure was particularly difficult, or that the authors needed to test other vectors or other transmembrane regions before selecting the conditions used. In fact, the entire procedure of amplifying out the GM-CSF encoding region from an expression library and cloning it into the pHOOK-1 vector is viewed as so straight-forward by the authors that it occupies only two paragraphs in small type in the methods section (page 7344, col. 1).

Soo Hoo et al. were apparently able to duplicate the procedure provided in the specification without undue experimentation. Accordingly, the specification is enabling for cells expressing cytokines in a membrane-associated form, even when the cytokine is normally secreted. Withdrawal of this rejection is respectfully requested.

- app was not adequately unpacked during examination

Rejections under 35 USC § 102:

Kimura et al.

Certain claims in the application stand rejected under § 102(a) as being anticipated by Kimura et al., Exp. Hematol. 24:360, 1996. The Office Action indicates that Kimura et al. teach a method of stimulating an immune response using a cellular composition comprising M-CSF (purportedly in the membrane-associated form), along with other features recited in the claims.

Applicants respectfully disagree. In the work described in the article, live L1210 tumor cells were administered into syngeneic mice, where they grew to a lethal size. When the cells were expressing M-CSF, the animals showed improved survival. There is no affirmative indication in the article that the M-CSF was present in a membrane associated form — in fact, it is referred to on page 361, col. 1 in soluble form (20 μg/kg), and levels of M-CSF of mice administered with M-CSF expressing cells was subsequently measured in serum (page 361, col. 2). It is also unclear whether the M-CSF expressing cells is being administered in combination with tumor antigen in a manner that elicits an immune response. Only survival data are given.

In contrast, Example 6 (page 61 ff.) of this application compares the immunogenic effect of cancer cells expressing a membrane-associated form of M-CSF, with cells expressing a secreted form of M-CSF. Animals receiving the membrane-associated form had considerably

better survival times than those receiving the secreted form (page 62, lines 22-25). Moreover, rejection of cells amongst various clones correlated with the level of membrane expression of M-CSF (page 62, line 25 to page 63, line 2). Evidence of a tumor-specific immune response was obtained by showing that the immunized animals were protected against a rechallenge with parental T9 glioma cells, but not mammary adenocarcinoma cells (page 63, lines 3-10). Furthermore, protection could be transferred from one animal to another by way of a lymphocyte-containing cell population (page 63, lines 11-17).

Claims 31, 50, and their dependents are further distinguished from the article by Kimura et al., because these claims require that the cell expressing the cytokine be inactivated to prevent proliferation. This feature is not taught or suggested in the article, since live cells were used. There is no suggestion in the article that the cells can be inactivated in such a way that they are unable to proliferate, but still remain viable and continue to synthesize the recombinant cytokine. In contrast, this patent application describes how the cells can be irradiated with a titrated dose to prevent proliferation, but still synthesize cytokine (page 47, line 15 to page 48, line 24; page 51, lines 14-22; page 53, lines 21-25; page 59, lines 14-23).

Claim 64 and its dependents are further distinguished from the article by Kimura et al., as discussed during the interview, because the claim requires that the cell expressing the cytokine be allogeneic to the treated subject. It is reasonable to surmise that the L1210 cells used in the article were syngeneic to the animals they were administered to, because they grew to form a tumor of lethal size without being rejected. Transfecting the cells with M-CSF apparently affected the viability or proliferation of the cells themselves in vivo. In contrast, the invention claimed in this patent application uses cytokine-expressing cells to recruit a host response against a tumor that may already be established in the host. In this manner, the immunizing cells are different from the cells against which the response is desired. The immunizing cells can be autologous or allogeneic to the subject being treated. In the embodiment of Claim 64, the immunizing cells are necessarily allogeneic.

Applicants do not concede that this publication is valid prior art under § 102(a). Applicants also do not concede that the article describes the features recited in the dependent claims in this application. These points will not be addressed further, because the arguments presented already are sufficient to overcome the rejection.

Jadus et al.

Certain claims in the application stand rejected under 35 USC § 102(a) as being anticipated by Jadus et al., Blood 87:5232, 1996. The Office Action indicates that Jadus et al. teach a method of killing tumor cells comprising administering a composition expressing M-CSF.

Applicants respectfully disagree. First of all, the reference does not qualify as § 102(a) prior art. The scientists in the Jadus group and the inventors on this patent application collaborated at U.C. Irvine in the preparation of some of the reagents used. Graf and Hiserodt are acknowledged in the article on page 5233, col. 1, paragraph 1. Jadus is acknowledged in the patent application on page 62, line 5. Glioma cell line T9 transduced with the LXSN retrovirus expression vector were used in both cases. Description of preparation of the cells on page 5233 of the article mirrors the description in the patent application on pages 61-62. The use of the cells in the article and in the patent application is directed to different purposes.

The article also differs from the claimed invention on a substantive basis. Jadus et al. report that macrophages killed hybridoma cells or T9 glioma cells expressing membrane M-CSF (Figure 5). The killing was inhibited by adding M-CSF to the medium (Table 2). The macrophage kill the cells directly (Figure 7), by binding M-CSF (macrophage colony stimulating factor) through receptors on the macrophage surface. This does not constitute administration of cytokine expressing cells to a subject. Instead, the cytokine expressing cells are used as target cells in a tissue culture experiment. Since the reaction consists of macrophages directly attacking target cells, it does not constitute an immunological response, which classically involves specific antibody or antigen-specific cells (T or B lymphocytes).

Claims 31, 50, and their dependents are further distinguished from the article by Jadus et al., because these claims require that the cell expressing the cytokine be inactivated to prevent proliferation. Claim 64 and its dependents are further distinguished from the article by Jadus et al., because it requires that the cell expressing the cytokine be allogeneic to the treated subject.

Withdrawal of all prior art rejections is respectfully requested.

Conclusion

Applicants respectfully request that all outstanding rejections be reconsidered and withdrawn. The application is believed to be in condition for allowance, and a prompt Notice of Allowance is requested.

In the event that the Examiner determines that there are other matters to be addressed, applicants hereby request an interview by telephone.

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number IRVN-001DIV2.

Respectfully submitted,
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Date: March 3, 2003

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Enclosure:

W. Soo Hoo et al., *Tumor cell surface expression of GM-CSF elicits antitumor immunity and protects from tumor challenge in the P815 mouse mastocytoma tumor model.* J. Immunol. 162:7343-7349, 1999

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Tumor Cell Surface Expression of Granulocyte-Macrophage Colony-Stimulating Factor Elicits Antitumor Immunity and Protects from Tumor Challenge in the P815 Mouse Mastocytoma Tumor Model

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A novel membrane-bound form of GM-CSF (mbGM-CSF) was expressed on the surface of the mouse mastocytoma cell line P815 to target tumor cell-associated Ags to epidermal Langerhans cells. Transfected clones stimulated the proliferation of syngeneic bone marrow cells, indicating that mbGM-CSF is biologically active. We evaluated the in vivo effects of mbGM-CSF by comparing the growth of mbGM-CSF cells (termed 1D6.1E5) to that of wild-type P815 cells in DBA/2 mice. The growth rates of tumors initiated by P815 and 1D6.1E5 were similar until day 12, after which P815 tumors grew to large sizes while 1D6.1E5 tumors were rejected. In contrast, the growth of both tumors was unimpeded when injected into nude mice, suggesting that a T cell-dependent antitumor response was induced by 1D6.1E5 in normal mice. Lymphocytes from 1D6.1E5-vaccinated mice were able to kill ^{51}Cr-labeled P815 cells in a dose-dependent fashion that was inhibited by anti-CD8 Abs, suggesting that the antitumor response involved CD8$^+$ CTL. We then tested whether vaccination with these cells would elicit a protective antitumor response by injecting mice with either irradiated 1D6.1E5 or P815 cells and challenging them with nonirradiated P815 cells. 1D6.1E5-treated mice grew small tumors that soon disappeared in all animals. In contrast, the majority of animals receiving the irradiated wild-type tumor vaccine grew large tumors, and 50% died. These data demonstrate that mbGM-CSF expressed on the surface of tumor cells is biologically active and elicits protective antitumor immunity. *The Journal of Immunology,* **1999, 162: 7343–7349.**

Preclinical tumor models using genetically modified tumor cells to secrete cytokines have been used in efforts to augment the immune response against tumor-associated Ags (1, 2). One of the more promising cytokines for the induction of potent antitumor activity is GM-CSF. This 24-kDa glycosylated cytokine has paracrine as well as autocrine effects on a number of cell types, including monocytes, dendritic cells, eosinophils, and neutrophils (3, 4). In preclinical and clinical studies, GM-CSF secreted from tumor cells has been shown to be a potent stimulator of antitumor responses (5–8). The consensus from these studies suggests that GM-CSF stimulates APC such as dendritic cells (DC)[2] to generate potent immune responses.

DC are the most potent APC in the immune system (9–11) and are able to prime naive T cells almost 30–100 times more efficiently than B cells (12, 13). Langerhans cells (LC) are immature DC that reside in the epidermis and continually sample Ag encountered in this compartment. Once LC receive the appropriate stimulus (e.g., GM-CSF, TNF-α, TGF-β, and LPS), they mature into DC (14) and migrate to lymph nodes, appearing within 24 h and peaking at 2 days after Ag uptake (15, 16), when they initiate the activation of naive T cells. These characteristics have made LC and DC the focus of intense research and attractive targets for immunotherapy. Immunotherapeutic approaches using DC include Ag pulsing of autologous DC (17, 18), transfection of DC with plasmids encoding Ags (19), and fusion of DC to tumor cells (20, 21). Taken together, these studies strongly suggest that to optimally induce an immune response using LC and DC, two requisites must be met. First, LC must be in close proximity to the appropriate Ag(s), and second, LC must receive the appropriate signals to cause maturation and migration of Ag-loaded cells to the lymph nodes to activate naive T cells.

We report here the expression and use of a novel form of GM-CSF anchored to the surface of the mouse P815 mastocytoma line through fusion with a heterologous transmembrane domain. We tested the hypothesis that tumor cells modified to express membrane-bound GM-CSF (mbGM-CSF) would effectively target tumor Ags to DC and provide an effective immune response against the unmodified parental tumor cells.

Materials and Methods

Mice

DBA/2 female mice, 8–10 wk old, and BALB/c *nu/nu* mice were purchased from Charles Rivers Laboratories (Wilmington, MA).

Antibodies

The following Abs were purchased from PharMingen (San Diego, CA). Anti-GM-CSF Ab MP1-22E9 (a rat anti-mouse GM-CSF mAb), anti-CD8 Ab 53-6.7, 28.14.8 (a mouse anti-L^d Ab), SF1-1.1 (a mouse anti-K^d Ab), 34-2-12 (a mouse anti-D^d Ab), and an isotype-matched control Ab IgG2a.

Cells

P815, a mouse (H-2^d) mastocytoma derived from the DBA/2 mouse strain, was a gift from Dr. David M. Kranz (University of Illinois, Urbana, IL).

The Immune Response Corporation, Carlsbad, CA 92008

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[2] Abbreviations used in this paper: DC, dendritic cells; mbGM-CSF, membrane-bound GM-CSF; LC, Langerhans cells; PDGFR, platelet-derived growth factor receptor; CD40L, CD40 ligand.

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Clones 1D1 and 1D6 are mbGM-CSF-positive cells derived from P815 transfected with the vector made as described below. The clone 1D6.1E5 is a subclone derived from the 1D6 cell line by limiting dilution cloning.

Plasmid vectors

For expression of an mbGM-CSF molecule, the pHOOK-1 plasmid vector was used for cloning (Invitrogen, Carlsbad, CA). Standard DNA cloning techniques were used for construction of the vectors.

Construction of mouse mbGM-CSF

The pHOOK-1 plasmid vector originally contained the coding sequence for a single-chain Ab located between the murine κ-chain signal peptide and the platelet-derived growth factor receptor (PDGFR) transmembrane domain coding sequences. The gene encoding the single-chain Ab was removed by cutting with restriction enzymes *ApaI* and *SalI*. The resulting vector was treated with calf intestinal alkaline phosphatase (Life Technologies, Gaithersburg, MD) to remove the terminal phosphate groups.

The cDNA of murine GM-CSF was derived from PCR using BALB/c mouse mRNA from spleen cells stimulated with 4 μg/ml Con A for 2 days. The cells were lysed in RNAzol (Life Technologies), and the total RNA was extracted. Oligo(dT) primers were used to prime the synthesis of cDNA from target mRNA. The following PCR primers were used with Taq polymerase in a standard PCR reaction using a Perkin-Elmer Thermocycler (Norwalk, CT): 5'Apa mseGM-CSF, 5'-GCTAGGGCCCTAGCAC CCACCCGCTCACCCATCACT-3'; and 3'Sal mseGM-CSF, 5'-AC CGCGGTCGACTTTTTGGACTGGTTTTTTGCATTCAAAGGGG-3'. The resulting PCR fragment was purified and cloned into compatible sites in pHOOK-1 using T4 ligase (Life Technologies).

Transfection of cells

Electroporation was used for transfecting the plasmid construct into P815. Briefly, cells were grown in log phase using standard tissue culture methods in RPMI 1640 supplemented with 10% FBS and antibiotics. Cells (5×10^6) were electroporated at a voltage of 250 V in the presence of 50 μg linearized plasmid vector. Cells were then incubated with 800 μg/ml G418 (Life Technologies), and subclones were screened by FACS for the presence of GM-CSF on the surface of the cells (as described below).

Flow cytometric assays

Cells (10^6) were washed once with 2% FBS in PBS. The cells were resuspended in 50 μl of wash buffer containing 40 μg/ml rat anti-mouse GM-CSF Ab MP1-22E9 and were incubated on ice for 30 min. Cells were washed twice with wash buffer and were resuspended in wash buffer containing a fluorescein-labeled secondary Ab (goat anti-rat, mouse absorbed; Kirkegaard & Perry Laboratories, Gaithersburg, MD) and were incubated on ice 30 min. After two washes with wash buffer, the cells were resuspended in 500 μl of PBS. In some experiments cells were fixed with 4% paraformaldehyde in PBS. For flow cytometric analysis, a Becton Dickinson FACSort was used (Becton Dickinson, San Jose, CA).

Bone marrow proliferation studies

P815 or clone 1D6.1E5 cells (10^7) were incubated with 250 μg/ml mitomycin C for 30 min at 37°C. The cells were extensively washed with PBS, pH 7.2, containing 5% (v/v) FBS, resuspended in RPMI 1640 medium supplemented with 10% FBS and antibiotics, and then added to round-bottom wells at the amounts indicated. In test wells, 3×10^4 DBA/2 bone marrow cells were added, and the cultures were incubated for 2 days at 37°C in 5% CO_2 in a humidified incubator. The wells were then pulsed with 1 μCi/well [^{3}H]thymidine and harvested the next day using a Cambridge Technology PHD cell harvester (Watertown, MA).

Evaluation of live tumor growth

Wild-type P815 cells (10^6) or mbGM-CSF clones at a concentration of 20×10^6 cells/ml were injected intradermally into the hind flanks of DBA/2 mice. In other experiments, tumor cells were injected intradermally into BALB/c *nu/nu* mice. Tumors were measured three times per week over the course of the experiment starting 7–10 days after the injections. Tumor sizes are expressed as a product of the longest diameter and the shortest diameter as measured by a calibrated micrometer. Five to ten mice per group were injected.

Evaluation of irradiated tumor vaccination

Wild-type P815 cells (10^6) or mbGM-CSF clone 1D6.1E5 were irradiated with 20,000 rad in the presence of complete tissue culture medium using a JLSheperd and Associates model 109-85 Irradiator with a ^{60}Co source.

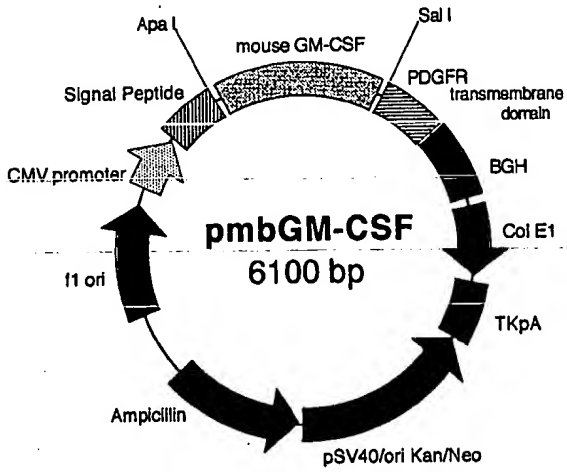

FIGURE 1. Construction of the mbGM-CSF plasmid vector. pHOOK-1 was the commercial plasmid vector into which the 372-nucleotide cDNA of GM-CSF was cloned. *ApaI* and *SalI* restriction endonuclease sites were added by PCR using primers indicated in *Materials and Methods*.

After irradiation, cells were washed with complete medium once, then extensively with PBS before injection into one flank of DBA/2 mice (10 mice/group). After 15 days the mice were boosted with the same number of cells in the same manner. Mice receiving irradiated wild-type cells were designated the control group. Five days after the boost, both groups of mice were challenged s.c. in the opposite flank with 10^6 live wild-type P815 cells. Tumors were measured as described above.

^{51}Cr release assays

Groups of five mice were vaccinated intradermally in the hind flank with 10^6 irradiated tumor cells with or without mbGM-CSF. Mice were boosted intradermally, and spleens were removed 5 days after the boost. Spleen cells were incubated with irradiated P815 cells at a ratio of 10:1 (spleen cells:tumor cells) for 5 days in complete medium. After stimulation with tumor cells, spleen cells were harvested, separated from dead cells and debris by Nycodenz density centrifugation (Accurate Chemical & Scientific Corp., Westbury, NY), then depleted of CD4$^+$ cells by Dynal magnetic bead separation (Dynal, Lake Success, NY). Target cells were prepared by adding 150 μCi of ^{51}Cr to 3×10^6 P815 cells in log phase growth for 1 h at 37°C with occasional agitation. The effector cells were then incubated at the indicated E:T cell ratios with ^{51}Cr-labeled P815 cells for 4 h in a humidified 37°C incubator. In Ab inhibition experiments, anti-CD8 Abs or a mixture of anti-class I Abs were used with the final concentrations of Abs indicated. Supernatants were then evaluated for ^{51}Cr release. The percent specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \left[\frac{(\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}})}{(\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}})}\right] \times 100.$$

Results

Construction and expression of a membrane-anchored GM-CSF gene

The mouse GM-CSF gene consisting of 372 nucleotides was amplified from cDNA derived from Con A-stimulated mouse splenocytes, placed under control of the CMV promoter downstream of a murine Ig κ-chain signal sequence, and fused to the sequence of the PDGFR transmembrane domain (Fig. 1). We took advantage of the commercial vector, pHOOK-1, which was originally designed to express a hapten-specific single-chain Ab anchored to the plasma membrane through the PDGFR transmembrane domain (22). Cells transfected with pmbGM-CSF were selected under drug treatment, and subclones were derived from limiting dilution cloning. FACS analysis demonstrated that mbGM-CSF was expressed at levels comparable to that of the endogenous class I molecule, K^d, while isotype-matched control Abs failed to stain cells (Fig. 2). For experiments in which transfected cells were irradiated to halt

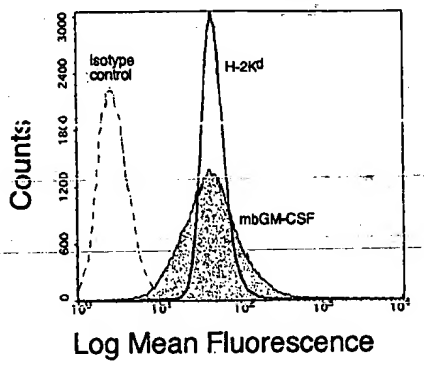

FIGURE 2. Surface expression of mbGM-CSF. P815 cells transfected with the pmbGM-CSF plasmid vector were stained with a mAb to mouse GM-CSF, anti-K^d, or isotype-matched (IgG2a) control Ab and analyzed by flow cytometry.

cell division, we observed that 20,000 rad did not alter the levels of mbGM-CSF on the surface of cells (data not shown). It is interesting to note that P815 cells express approximately 10^3 GM-CSF receptors/cell (23); however, we did not observe any significant coagulation or cell-cell clumping compared with those of wild-type cells (W.S.H. and K.A.L., unpublished observations).

mbGM-CSF cells stimulate DBA/2 bone marrow cells to proliferate in an in vitro proliferation assay

One of the important effects of GM-CSF is the stimulation of DC, resulting in their maturation marked by a rapid change from an Ag-sampling cell to APC. For efficient delivery of Ag to naive T cells, we believe it necessary for mbGM-CSF to be biologically active. Therefore, we determined whether mbGM-CSF would be able to mediate a signal to cells that are normally responsive to soluble GM-CSF. We chose to stimulate syngeneic bone marrow cells, which express the GM-CSF receptor and proliferate in the presence of soluble GM-CSF in a dose-dependent manner (24). In Fig. 3, cells expressing mbGM-CSF stimulated the proliferation of bone marrow cells, while the control wild-type P815 cells did not (Fig. 3*A*). This stimulation was specifically inhibited by the addition of an anti-GM-CSF mAb in a dose-dependent manner, whereas an isotype control Ab had no effect (Fig. 3*B*). GM-CSF also could be detected by ELISA at low levels in supernatants of membrane-bound clones (<8 pg/10^6 cells/24 h). However, these levels were orders of magnitude below that required for stimulation of bone marrow cells and approximately 4000-fold less than that reported to be biologically relevant in the elicitation of an antitumor immune response in mouse tumor models (25).

Growth rates of P815 vs 1D6.1E5 cells are unimpeded in vitro and in vivo in an immunocompromised host

We wanted to ensure that any differences we might observe in tumor growth in in vivo experiments were not due to differences in the intrinsic growth rates of the transfected cells vs wild-type cells. Therefore, the growth rates of both wild-type P815 and transfected clones were compared in vitro. Fig. 4*A* shows that the growth rates of 1D6.1E5 cells (a subclone of 1D6) were essentially the same as those of wild-type cells. Also, it made no difference whether the transfected cells were growing in the presence or the absence of the G418 selection drug. Growth rates were also determined to be equal in T cell-deficient BALB/c *nu/nu* nude mice (Fig. 4*B*). These data suggest that in the absence of a significant T cell response, cells expressing mbGM-CSF grow as solid tumors at the same rate as wild-type cells. An observation was made in all the in vivo studies with both nude and normal DBA/2 mice that there was a characteristic reduction in the mean tumor size of mice after 18–20 days. This apparent reduction was the result of the death of mice with the largest tumor burdens. We noted that the mean values of tumor size rose again, which represented the continued growth of tumors in the remaining mice.

In vivo tumor rejection of live mbGM-CSF cells in DBA/2 mice

Normally, P815 cells will grow as solid tumors when injected into a syngeneic host. It was of interest to determine whether there would be a difference between the growth of tumors initiated by transfected clones and wild-type cells. Nonirradiated P815 cells or clones bearing mbGM-CSF (designated 1D1 and 1D6) were injected intradermally into the flanks of DBA/2 mice. Initially, both mbGM-CSF cells and wild-type P815 cells grew similarly in the host. However, on day 12, tumors caused by the mbGM-CSF cells were quickly rejected, while wild-type P815 cells produced tumors that grew to significantly large sizes (Fig. 5). Indeed, for clone 1D6, 100% of animals became tumor free, while clone 1D1 showed low, but measurable, numbers of tumors. These animals were later challenged with live wild-type P815 in the opposite

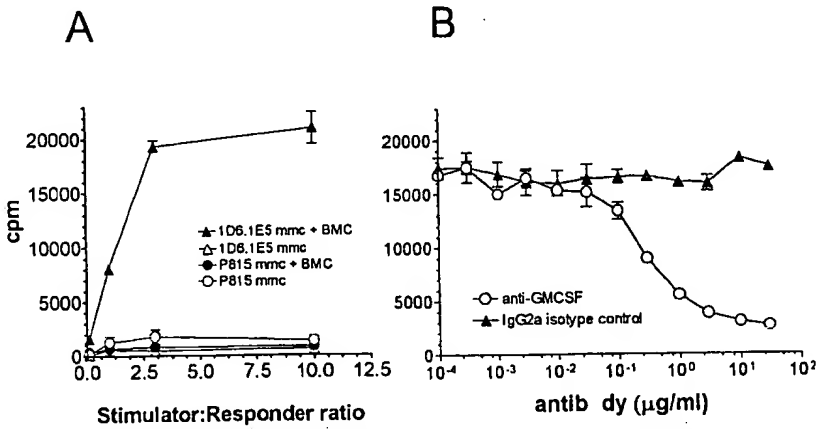

FIGURE 3. In vitro assessment of the biological activity of mbGM-CSF tumor cells. *A*, Mitomycin C-treated (mmc) stimulator cells (solid triangle, 1D6.1E5 cells; solid circle, P815 cells) were incubated in a [^{3}H]thymidine proliferation assay in the presence of 3×10^4 DBA/2 bone marrow cells (BMC) in the ratios indicated. Mitomycin C-treated stimulator cells were also cultured without BMC to control for background counts per minute (open triangle, 1D6.1E5 cells alone; open circle, P815 cells alone). *B*, BMC were stimulated by mitomycin C-treated 1D6.1E5 cells at a ratio of 3:1 (1D6.1E5: BMC) in the presence of increasing amounts of anti-GM-CSF or isotype-matched control Abs.

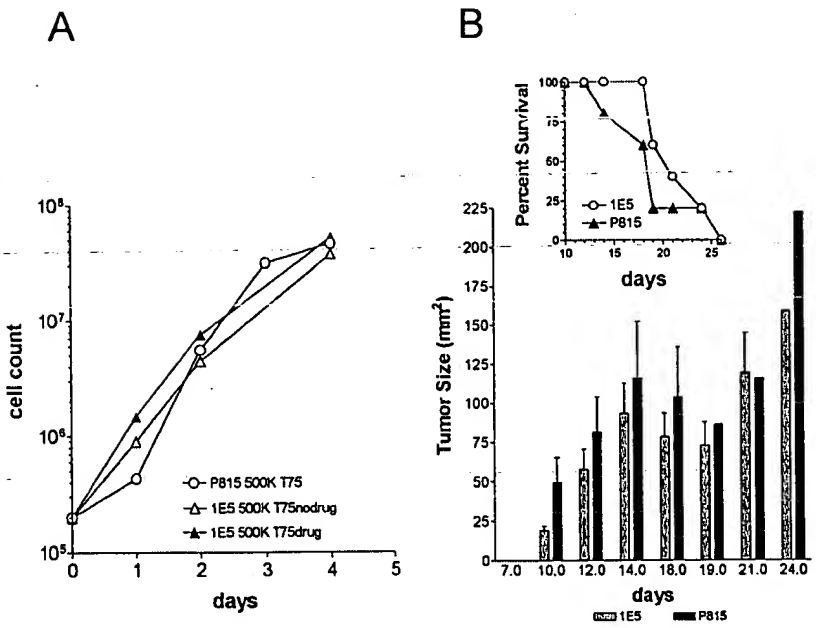

FIGURE 4. Growth comparisons of mbGM-CSF clones with wild-type P815 cells. *A*, Cells were seeded with complete medium at 5×10^5 cells/well in 75-cm^2 culture flasks and counted on the indicated days. *B*, 1D6.1E5 cells or P815 cells (1×10^6) were injected i.d. into BALB/c nude mice (five mice per group) and observed for tumor growth and survival.

flank and were able to reject these tumors. It was interesting that the FACS comparisons between these two clones showed that clone 1D6 expressed a significantly higher density of mbGM-CSF than did 1D1 (data not shown), which may explain the differences observed between the responses to 1D6 and 1D1 injections. For these reasons, we continued experiments with a subclone of 1D6 termed 1D6.1E5. Preliminary experiments from our laboratory using a transfected P815 cell line expressing a membrane-bound form of IL-4 (associated with a Th2 cytokine response) demonstrated that mbIL-4-expressing cells were not rejected as efficiently as cells expressing mbGM-CSF (W. Soo Hoo and J. R. Kohrumel, unpublished observations).

Vaccination with irradiated clones yields protection from a wild-type tumor challenge

Prevention of the growth of nonirradiated tumor cells may be a function of innate immunity responding to the proinflammatory effects of GM-CSF as opposed to the longer lasting effects of an adaptive immune response. To evaluate whether the mbGM-CSF-expressing cells (1D6.1E5) could elicit a protective and systemic antitumor response, irradiated cells were used to vaccinate mice before challenge with nonirradiated wild-type P815 cells. DBA/2 mice were injected intradermally with 10^6 cells in the left flank and boosted 15 days later with the same number of cells in the same flank. Five days after the last vaccination, the mice were challenged s.c. with nonirradiated wild-type tumor cells in the opposite flank. Although all mice developed palpable tumors in the first 2 wk, only animals vaccinated with mbGM-CSF cells were able to reject their tumors completely. By 30 days after the initial challenge with wild-type tumor cells, the treated group did not show any signs of tumor growth and remained tumor free throughout the remainder of the experiment. The control group, however, grew large tumors, and 50% of the mice died (Fig. 6, inset). In another experiment, mice were prevaccinated with half the dose (5×10^5 cells), and a group was added that received no vaccination before

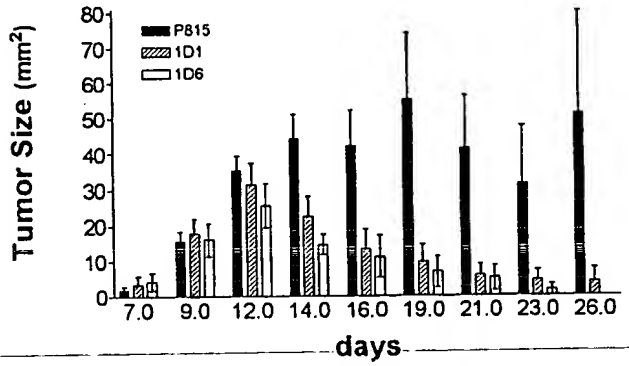

FIGURE 5. In vivo rejection of mbGM-CSF cells by syngeneic hosts. DBA/2 mice (10 mice/group) were injected with 10^6 cells intradermally in the hind flank. Tumor sizes were measured on the indicated days. Filled bars, wild-type P815; shaded bars, clone 1D1; open bars, clone 1D6.

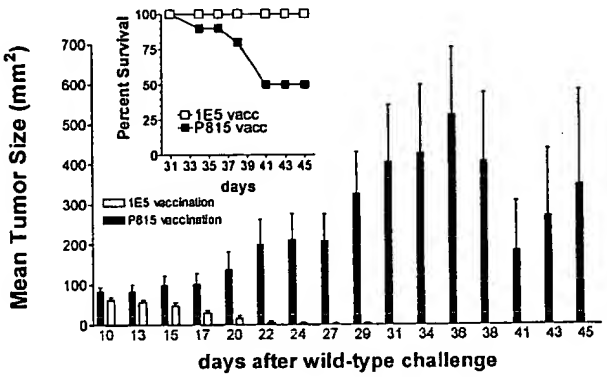

FIGURE 6. Vaccination with mbGM-CSF cells protects mice from a wild-type tumor challenge. DBA/2 mice (10 mice/group) were prevaccinated with 10^6 irradiated 1D6.1E5 or P815 cells intradermally in one hind flank. Five days after boosting in the same flank, animals were challenged s.c. with 10^6 nonirradiated P815 cells in the opposite flank. Tumors and survival were observed on the indicated days. Filled bars, P815 vaccination; open bars, 1D6.1E5 vaccination.

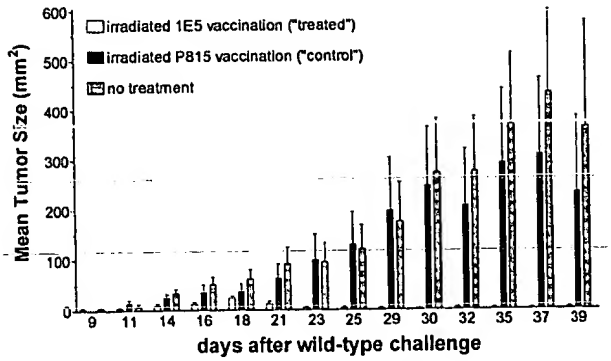

FIGURE 7. Vaccination with low doses of mbGM-CSF provides protection against a challenge of wild-type tumor cells. Irradiated 1D6.1E5 or P815 cells (5×10^5) were injected intradermally into mice (10 mice/group) and boosted. Five days later, mice were challenged s.c. with 10^6 nonirradiated P815 cells in the opposite flank, and tumors were observed on the indicated days. Open bars, 1D6.1E5 vaccination; filled bars, P815 vaccination; shaded bars, no prevaccination.

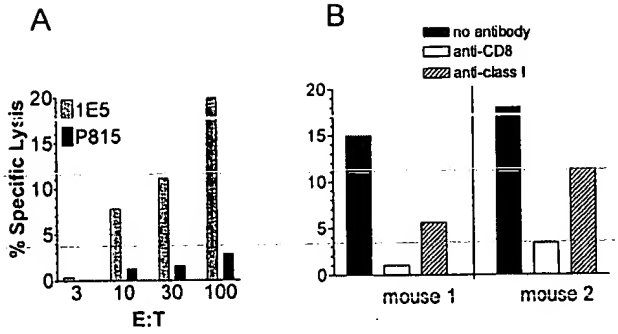

FIGURE 8. Vaccination with 1D6.1E5 cells generates wild-type tumor-specific CTL that are inhibited by anti-CD8 and anti-class I Abs. DBA/2 mice were vaccinated with 10^6 irradiated 1D6.1E5 or P815 cells and boosted on day 14. Five days after boosting, splenocytes were prepared and stimulated in vitro for 5 days in the presence of irradiated P815 cells. *A*, CTL were cultured in the presence of ^{51}Cr-labeled P815 cells in a standard 4-h chromium release assay at the indicated E:T cell ratios. *B*, Mice were primed and boosted as described above and CTL killing (E:T cell ratio, 30:1) was inhibited by specific Abs. Filled bars, no Ab added; open bars, anti-CD8 Abs (125 μg/ml); shaded bars, anti-L^d, anti-K^d, anti-D^d (each at 42 μg/ml).

challenge. Once again, the majority of mice vaccinated with the mbGM-CSF cells were able to completely reject their tumors, while no significant difference was observed between either the mice vaccinated with wild-type P815 cells or those receiving no vaccination (Fig. 7).

Antitumor activity is mediated by CTLs

In light of the results of tumor growth in nude mice and the kinetics of tumor rejection in other experiments, it seemed reasonable to assume that the antitumor activity observed in normal mice was due in large part to a proliferation of tumor-specific T cells. To test this idea, mice were immunized with 10^6 irradiated mbGM-CSF clone 1D6.1E5 or wild-type P815 cells. Five days after a boost with the same number of cells, splenocytes were isolated from all mice and given one round of stimulation by irradiated wild-type P815 cells. The question was then asked whether CTL from these preparations could kill wild-type P815 tumor cells. In Fig. 8*A*, mice that have been vaccinated with mbGM-CSF cells produce significantly more anti-P815 CTLs than mice receiving the wild-type vaccination. Anti-CD8 Abs (at a final concentration of 125 μg/ml) could inhibit the specific killing (Fig. 8*B*), indicating that the killing was $CD8^+$ CTL dependent. Furthermore, when a mixture of anti-class I Abs (L^d, K^d, D^d, each at 42 μg/ml final concentration) was used, they also inhibited the killing of P815 target cells, although to a slightly lesser degree (Fig. 8*B*). Isotype control Abs failed to inhibit CTL killing of P815 targets (data not shown). In a number of experiments, the mbGM-CSF cells consistently elicited CTL in a manner superior to P815 wild-type cells (Table I).

Discussion

To our knowledge, this is the first report of a soluble cytokine genetically engineered to be expressed in a membrane-bound form. There are examples of cytokines expressed as membrane-anchored proteins; however, these represent natural alternative splice forms (Refs. 26 and 27; reviewed in Ref. 28). Earlier work by Tao and Levy showed that GM-CSF fused to a single protein Ag was a potent immunogen and that the GM-CSF component of the fusion protein was biologically active (29). Also, the three-dimensional structure of GM-CSF described by Diedrichs et al. suggested that the molecule could be fused through its carboxyl terminus without hindering the putative receptor contact points (30). In light of this, we thought it reasonable that anchoring the GM-CSF molecule to the cell surface could be accomplished while maintaining its biological activity. Thus, we have taken advantage of the high affinity interaction between GM-CSF and its cognate receptor CD116/CDw131 (K_d = 120 pM) (31) to target tumor Ags to epidermal LC through direct cell-cell interaction.

In this study we have demonstrated that a novel mbGM-CSF on the surface of the P815 mastocytoma cell line can generate an antitumor immune response in syngeneic DBA/2 hosts. The

Table I. *Data from three CTL assays from mice vaccinated with P815 cells with or without mbGM-CSF*[a]

	% Specific Lysis									
	3:1[b]		10:1[b]		30:1[b]		75:1[b]		100:1[b]	
Expt.	1D6.1E5	P815	1D6.1E5	P815	1D6.1E5	P815	1D6.1E5	P815	1D6.1E5	P815
I[c]	15.0	5.0	27.0	9.0	37.0	15.0	ND	ND	ND	ND
II[c]	6.5	3.0	16.0	6.7	30.0	11.0	61.0	28.0	ND	ND
III[d]	0.4	0.1	7.8	1.3	11.2	1.6	ND	ND	20.0	2.9

[a] The % specific lysis is given for each E:T ratio and for each vaccination.
[b] P815 tumor cells labeled with ^{51}Cr were used as targets in all assays.
[c] T cell cultures derived from animals vaccinated with P815 cells were stimulated in vitro with irradiated P815 cells while T cell cultures derived from animals vaccinated with 1D6.1E5 cells were stimulated in vitro with irradiated 1D6.1E5 cells.
[d] T cell cultures were stimulated in vitro with irradiated P815 cells regardless of the cells used for vaccination.

mbGM-CSF molecules are expressed at levels comparable to those of class I molecules (Fig. 2) and are able to stimulate bone marrow cells in an in vitro analysis demonstrating that the membrane-bound molecules have retained biological activity (Fig. 3). Based on our assays using soluble recombinant mouse GM-CSF, we calculate that a response yielding a stimulation index of 20 was equivalent to 2.6×10^{10} molecules of soluble GM-CSF. The number of 1D6.1E5 cells that produced the equivalent stimulation was 3×10^5 cells. Assuming that the activity/binding affinity of mbGM-CSF is the same as that of soluble GM-CSF and that each mbGM-CSF molecule on the surface has an opportunity to bind receptor, we estimate that there are 10^5 molecules of mbGM-CSF/cell. In studies using ^{125}I-radiolabeled anti-L^d mAbs, the P815 tumor line expressed approximately 6×10^5 molecules of L^d on the surface (40).

The P815 tumor cell line is known to be moderately immunogenic in the syngeneic host (32), and repeated vaccinations with wild-type P815 cells result in some antitumor immunity. For this reason, we determined that the most appropriate controls for these studies are mice vaccinated with wild-type cells. In the experiment in which nonirradiated mbGM-CSF P815 cells were injected into mice, tumors grew during the first 10–12 days. After this time, however, the tumors were quickly rejected until no palpable tumor was detected (Fig. 5), while wild-type cells grew to large sizes, resulting in 50% mortality. One explanation for this initial growth is that the priming of the immune response through the interaction of GM-CSF and DC results in a short lag period before sensitized CTL can be generated in sufficient numbers. In contrast, Nakajima et al. demonstrated that rejection of P815 cells transfected to express CD40L was immediate (i.e., no tumor growth was observed), and they showed that this rejection was due to a significant contribution of NK cells (33). When the mbGM-CSF cells were injected into BALB/c nude mice, the transfected cells grew at the same rate as wild-type cells. This is in contrast to the results seen in the Nakajima report in which CD40L-P815 cells suppressed tumor growth in nude mice, suggesting that the mechanism of antitumor immunity using mbGM-CSF is different from that used by the CD40-CD40L system. Our results are in agreement with those obtained by Sampson et al., who reported that the contribution of NK cell activity elicited by soluble GM-CSF from modified B16 melanoma cells is measurable, but relatively minor (6). This further suggests that the immunity elicited by mbGM-CSF cells is T cell dependent and that very little, if any, innate immunity is involved.

The rejection of live mbGM-CSF cells demonstrated the elicitation of an immune response directed to the modified tumor cells. This is in contrast to an initial observation in a study by Dranoff et al. in which they report that injection of live tumor cells secreting soluble GM-CSF grew progressively, inducing lethal toxicity and hepatosplenomegaly (5). However, when used as an irradiated vaccine, the GM-CSF-secreting cells induced strong antitumor immunity. From this observation one might speculate that the mbGM-CSF may be safe, since injection of live cells did not produce any lethal toxicity. Taken together with the data we have shown in athymic mice (Fig. 4*B*), we hypothesized that the rejection of live mbGM-CSF cells is mediated by a T cell-dependent systemic immune response and that this response to 1D6.1E5 cells may extend to the parental P815 cells.

The final goal of this study was to show that CTL generated using a vaccine composed of irradiated mbGM-CSF cells could elicit an immune response directed to the unmodified, parental tumor cell line both in vitro and in vivo. When used as a cell vaccine, the mbGM-CSF P815 cells were consistently superior to similar vaccinations of wild-type P815 cells in the elicitation of tumor-specific CTL (Fig. 8 and Table I) against the challenge of unmodified tumor cells. These observations are in agreement with the idea that GM-CSF is a potent cytokine adjuvant for the elicitation of antitumor responses. Since the major tumor Ags (e.g., p1A and p2Ca) of P815 are known, we plan to examine the specificity of CTL generated to the mbGM-CSF P815 cells with regard to their recognition of specific peptide Ags. The demonstration of tumor-specific CTL does not rule out the possibility that a relevant humoral immune response was also elicited. Future studies will investigate whether cells or sera from immunized animals can be adoptively transfered to convey protection from a tumor challenge or induce eradication of established tumors.

The mbGM-CSF differs from the current cytokine secretion paradigms in two distinct ways. First, contact with mbGM-CSF by GM-CSF receptors on dendritic cells requires direct physical contact with the cellular vaccine. In strategies where cytokines are secreted into the extracellular milieu, it may be tempting to speculate that DC would receive maturation signals coming from a gradient concentration of soluble GM-CSF without being close enough to take up specific Ags optimally. Also, it has been noted by others that the use of secreted, soluble cytokines requires relatively high rates of secretion for prolonged periods (~36 ng/10^6 cells/24 h) (24). Depending on the mode of transfection and the clones isolated, this may prove to be a serious limitation. The second distinction of this strategy lies in the ability to engage multiple GM-CSF receptors on a given DC. Stimulation of LC with GM-CSF causes maturation of LC in culture (34–36), resulting in the up-regulation of costimulatory molecules and increased expression of class I and class II molecules (37, 38). An additional signal is provided by the local production of TNF-α, which is responsible for the rapid migration of these cells to lymph nodes (39). We postulated that a high avidity cell-cell contact with multiple GM-CSF receptors on LC would cause the LC to experience a stronger signal transduction via the GM-CSF receptor α- and β-chains for efficient Ag uptake and rapid migration to lymph nodes. One of the aims of this system was to optimize the transduction of the signal that leads to the maturation and migration of DC. By increasing the avidity (i.e., the number of receptor-ligand interactions) between the tumor cell and the APC, we propose that the signal transduction to the DC is optimized.

The data shown here provide evidence that specific, systemic antitumor immunity can be elicited by tumor cells expressing GM-CSF on their surface. We believe that the adjuvant effect provided by mbGM-CSF is a result of the Ag-presenting DC in physical contact with the source of Ag (tumor cell), thus efficiently stimulating the antitumor response. Whether mbGM-CSF is more efficacious than secreted GM-CSF remains to be tested, and we are conducting experiments in other tumor models to directly compare the membrane-bound and secreted forms of GM-CSF.

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